

PCT

10/075,869
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, C07K 14/35, C12N 15/31		A2	(11) International Publication Number: WO 97/41252 (43) International Publication Date: 6 November 1997 (06.11.97)
(21) International Application Number: PCT/EP97/01973 (22) International Filing Date: 18 April 1997 (18.04.97) (30) Priority Data: 196 17 184.9 29 April 1996 (29.04.96) DE (71) Applicant (for all designated States except US): GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) [DE/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): SINGH, Mahavir [DE/DE]; (DE). HONISCH, Christiane [DE/DE]; (DE). ESPITIA, Clara [-/DE]; (DE). MORENO, Carlos [-/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). (74) Agents: BOETERS, Hans, D. et al.; Boeters & Bauer, Bereiteranger 15, D-81541 München (DE).			(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: DNA, RNA AND A PROTEIN USEFUL FOR DETECTION OF A MYCOBACTERIAL INFECTION			
(57) Abstract The invention concerns a DNA, RNA and a protein useful for identifying and combating mycobacterial infections.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Description

DNA, RNA AND A PROTEIN USEFUL FOR DETECTION OF A MYCOBACTERIAL INFECTION

Description

Technical field

The invention is in the field of clinical medicine, molecular biology and genetic engineering. More particularly, it relates to the molecular methods of tuberculosis diagnosis using newly identified DNA sequences which can be used as probes for DNA hybridization and or for DNA amplification leading to the identification of pathogenic mycobacteria causing disease in humans and animals.

Background

Tuberculosis, an infectious disease mainly caused by respiratory infection with *Mycobacterium tuberculosis*, represents an important subject of multidisciplinary investigation owing to the urgent need for rapid and reliable diagnostic tests and effective vaccines for disease control.

An estimated 8 million persons are developing tuberculosis each year and this number will be rising for the foreseeable future. Especially immuno-compromised people, e.g. Human Immunodeficiency Virus-infected individuals (Selwyn et al., 1989; Barnes et al., 1991) and the population of countries with

insufficient public health systems (Grzybowski, 1991; Kochi, 1991) are the most endangered groups of this "global disease" (WHO, 1992). Emergence of multiple drug resistant strains is posing major threat to human health not only in developing countries, but also in developed countries. A rapid and specific diagnosis of tuberculosis is still a problem.

One approach to address this problem is to use the specific humoral or cellular response of the host to infer the presence of disease. Mycobacteria are rich in antigens that stimulate the production of antibodies and serology is simple and readily applicable as a rapid diagnostic test (Wilkins, 1994). Unfortunately the usefulness of serological tests are often limited by their lack of specificity and by their inability to distinguish between active disease, prior sensitization by contact with *M. tuberculosis* or cross-sensitization to other mycobacteria.

Another means of achieving the correct diagnosis are to develop increasingly sensitive methods to detect the causative bacilli or their products. Such techniques include amplification of a defined region of bacterial DNA via polymerase chain reaction (PCR) (Shankar et al., 1991), immunoassays for detecting antigen, gas liquid chromatography and mass spectrometry for detecting specific mycobacterial lipids. Of these, PCR is being evaluated most intensely and appears to hold greatest promise.

Attempts have been made to develop methods for the detection of chromosomal DNA of the *M. tuberculosis* complex in patient's sputum (Glennon, 1994). While the possibility of developing a DNA probe to distinguish between the *M. tuberculosis* complex and other mycobacterial strains has been reported, strain differentiation within the individual members of the complex is still a problem.

In this study we report the isolation of novel genomic clones containing as yet unreported genes and DNA, and the identification of novel *M. tuberculosis* chromosomal DNA regions specific for species of the *M. tuberculosis* complex. In addition, amplification of (i) a 377 bp fragment specific for the *M. tuberculosis* complex and (ii) of a 380-bp fragment showing sequence similarities with the genome of *Mycobacterium asiaticum*, *Mycobacterium gastri*, *Mycobacterium gordonae* and *Mycobacterium kansasii* are described. The utility of the 377-bp and the 380-bp fragment for the differentiation of species and strains of mycobacteria is reported. In addition to other ORF identified in this study, a novel ca. 15kDa recombinant protein showing high homology to a family of transposase was overproduced in *Escherichia coli* as a thioredoxin fusion and purified. The ca. 15kDa and ca. 31kDa proteins described in this study are different from the 35kDa ORF belonging to an insertion element identified by Mariani et al. (1993).

Disclosure of invention

The present invention is based on novel DNA sequences cloned from the genome of *Mycobacterium tuberculosis*, which can be used for strain differentiation and for the diagnosis of tuberculosis.

Accordingly, the DNA sequences of the cloned fragments is an aspect of the invention.

The cloned DNA fragments are found to code for at least 7 proteins of about 9kDa, 15kDa, 17kDa, 31kDa, 55kDa, 74kDa and 77kDa, the sequences of which are another aspect of the invention.

The use of the DNA sequence for detecting specific fragments by hybridization or by DNA amplification is another aspect of the invention.

The use of the cloned DNA or of the proteins coded by the cloned DNA for the purpose of serology, skin testing, vaccine development or drug design is another aspect of the invention.

The object underlying the invention is solved by the following three main embodiments with their preferred embodiments.

According to a first embodiment the invention concerns a DNA

- (a) having sequence (I) according to figure 9, wherein optionally one or more condons can be replaced by condons coding for the same amino acid(s),
- (b) having a sequence complementary to said of (a),
- (c) being single-stranded, wherein its strand is hybridizable with that of the DNA according to (a) or (b),
- (d) being double-stranded, the sequences of its single strands being defined as in (a) and (b), respectively,
- (e) being double-stranded, its single strands being hybridizable with those of the DNA according to (d), or
- (f) being a subsequence of the sequences according to (a) to (e).

Further the invention concerns a DNA according to (c) or (e), its single strands being hybridizable with those of the DNA according to (a), (b), (d) and (f), respectively, at a temperature of at least 25 °C and at a concentration of NaCl of 1 M.

Further the invention concerns a RNA being a transcript of a DNA according to the invention.

Further the invention concerns a protein being encoded by a DNA according to the invention.

Further the invention concerns a protein having the amino acid sequence (II) according to figure 13.

The protein according to the invention can be an about 74 kDa protein.

Further the invention concerns a protein having the amino acid sequence (III) according to figure 14.

The protein according to the invention can be an about 77 kDa protein.

Further the invention concerns a protein having the amino acid sequence (IV) according to figure 15.

The protein according to the invention can be an about 9 kDa protein.

Further the invention concerns a protein having the amino acid sequence (V) according to figure 16.

The protein according to the invention can be an about 55 kDa protein.

The protein according to the invention can be a recombinant protein, especially a protein produced by means of a bacterial strain, a yeast strain, a fungal strain or a cell line of a higher eucaryote.

The protein according to the invention can be encoded by a DNA sequence according to the first embodiment of the invention and can be recovered by a method comprising the following steps:

- (i) subjecting proteins encoded by said DNA sequence to a usual test for diagnosis of tuberculosis,
- (ii) selecting a protein showing an inhibitory effect and
- (iii) isolating and recovering said protein.

Further the invention concerns a DNA, RNA or protein according to the invention which can be used for

- (i) diagnosis of tuberculosis in humans and animals and/or

(ii) diagnosis of other mycobacterial infections in humans or animals,
each especially by means of samples taken from humans or animals.

Further the invention concerns a use of a DNA according to the invention for the identification of mycobacteria in media samples.

The foregoing use can comprise the steps of

- (i) isolating the mycobacterium,
- (ii) preparing crude or purified genomic DNA,
- (iii) hybridizing it to a DNA according to the first embodiment of the invention and
- (iv) detecting the fragment pattern using conventional methods such as a radioactivity assay, chemiluminiscence or fluorescence.

Further the invention concerns a use, wherein as samples clinical samples are used.

Further the invention concerns a use of a DNA according to the invention or of a protein according to the invention for

- (i) epidemeological purposes and/or
 - (ii) vaccination follow-up
- for humans or animals suffering from mycobacterial infections, especially tuberculosis.

Further the invention concerns a use of a DNA according to the invention or of a protein according to the invention for the development of drugs useful for combating mycobacterial infections of humans or animals, especially tuberculosis, especially for testing and recovering of substances inhibiting mycobacterial infections in humans and animals, especially tuberculosis.

According to a second embodiment the invention concerns a DNA

- (a) having sequence (VI) according to figure 2, wherein optionally one or more condons can be replaced by condons coding for the same amino acid(s),
- (b) having a sequence complementary to said of (a),
- (c) being single-stranded, wherein its strand is hybridizable with that of the DNA according to (a) or (b),
- (d) being double-stranded, the sequences of its single strands being defined as in (a) and (b), respectively,
- (e) being double-stranded, its single strands being hybridizable with those of the DNA according to (d), or
- (f) being a subsequence of the sequences according to (a) to (e).

Further the invention concerns a DNA according to (c) or (e), its single strands being hybridizable with those of the DNA according to (a), (b), (d) and (f), respectively, at a temperature of at least 25 °C and at a concentration of NaCl of 1 M.

Further the invention concerns a RNA being a transcript of a DNA according to the invention.

Further the invention concerns a protein being encoded by a DNA according to the invention.

Further the invention concerns a protein having the amino acid sequence (VII) according to figure 5.

The protein according to the invention can be an about 15 kDa protein.

Further the invention concerns a protein having the amino acid sequence (VIII) according to figure 6.

The protein according to the invention can be an about 31 kDa protein.

The protein according to the invention can be a recombinant protein, especially a protein produced by means of a bacterial strain, a yeast strain, a fungal strain or a cell line of a higher eucaryote.

The protein according to the invention can be encoded by a DNA sequence according to the second embodiment of the invention and can be recovered by a method comprising the following steps:

- (i) subjecting proteins encoded by said DNA sequence to a usual test for diagnosis of tuberculosis,
- (ii) selecting a protein showing an inhibitory effect and
- (iii) isolating and recovering said protein.

Further the invention concerns a DNA, RNA or protein according to the invention which can be used for

- (i) diagnosis of tuberculosis in humans and animals and/or
 - (ii) diagnosis of other mycobacterial infections in humans or animals,
- each especially by means of samples taken from humans or animals.

Further the invention concerns a use of a DNA according to the invention for the identification of mycobacteria in media samples.

The foregoing use can comprise the steps of

- (i) isolating the mycobacterium,
- (ii) preparing crude or purified genomic DNA,
- (iii) hybridizing it to a DNA according to the second embodiment of the invention and

(iv) detecting the fragment pattern using conventional methods such as a radioactivity assay, chemiluminiscence or fluorescence.

Further the invention concerns a use, wherein as samples clinical samples are used.

Further the invention concerns a use of a DNA according to the invention or of a protein according to the invention for

(i) epidemeological purposes and/or

(ii) vaccination follow-up

for humans or animals suffering from mycobacterial infections, especially tuberculosis.

Further the invention concerns a use of a DNA according to the invention or of a protein according to the invention for the development of drugs useful for combating mycobacterial infections of humans or animals, especially tuberculosis, especially for testing and recovering of substances inhibiting mycobacterial infections in humans and animals, especially tuberculosis.

According to a third embodiment the invention concerns a DNA
(a) having sequence (IX) according to figure 3, wherein optionally one or more condons can be replaced by condons coding for the same amino acid(s),

(b) having a sequence complementary to said of (a),

(c) being single-stranded, wherein its strand is hybridizable with that of the DNA according to (a) or (b),

(d) being double-stranded, the sequences of its single strands being defined as in (a) and (b), respectively,

(e) being double-stranded, its single strands being hybridizable with those of the DNA according to (d), or

(f) being a subsequence of the sequences according to (a) to (e).

Further the invention concerns a DNA according to (c) or (e), its single strands being hybridizable with those of the DNA

according to (a), (b), (d) and (f), respectively, at a temperature of at least 25 °C and at a concentration of NaCl of 1 M.

Further the invention concerns a RNA being a transcript of a DNA according to the invention.

Further the invention concerns a protein being encoded by a DNA according to the invention.

Further the invention concerns a protein having the amino acid sequence (X) according to figure 7.

The protein according to the invention can be an about 17 kDa protein.

The protein according to the invention can be a recombinant protein, especially a protein produced by means of a bacterial strain, a yeast strain, a fungicidal strain or a cell line of a higher eucaryote.

The protein according to the invention can be encoded by a DNA sequence according to the third embodiment of the invention and can be recovered by a method comprising the following steps:

- (i) subjecting proteins encoded by said DNA sequence to a usual test for diagnosis of tuberculosis,
- (ii) selecting a protein showing an inhibitory effect and
- (iii) isolating and recovering said protein.

Further the invention concerns a DNA, RNA or protein according to the invention which can be used for

- (i) diagnosis of tuberculosis in humans and animals and/or
- (ii) diagnosis of other mycobacterial infections in humans or animals,

each especially by means of samples taken from humans or animals.

Further the invention concerns a use of a DNA according to the invention for the identification of mycobacteria in media samples.

The foregoing use can comprise the steps of

- (i) isolating the mycobacterium,
- (ii) preparing crude or purified genomic DNA,
- (iii) hybridizing it to a DNA according to the third embodiment of the invention and
- (iv) detecting the fragment pattern using conventional methods such as a radioactivity assay, chemiluminiscence or fluorescence.

Further the invention concerns a use, wherein as samples clinical samples are used.

Further the invention concerns a use of a DNA according to the invention or of a protein according to the invention for

- (i) epidemiological purposes and/or
 - (ii) vaccination follow-up
- for humans or animals suffering from mycobacterial infections, especially tuberculosis.

Further the invention concerns a use of a DNA according to the invention or of a protein according to the invention for the development of drugs useful for combating mycobacterial infections of humans or animals, especially tuberculosis, especially for testing and recovering of substances inhibiting mycobacterial infections in humans and animals, especially tuberculosis.

The invention is explained in detail by the following figures and experimental data.

Fig. 1 shows a restriction endonuclease map of the 7.2 kb *M. tuberculosis* chromosomal region;

Fig. 2. shows a 2253 bp *M. tuberculosis* chromosomal region including BamHI, EcoRI and KpnI restriction sites and oligonucleotides for screening the lambda gt 11 *M. tuberculosis* library (Primer 1 and Primer 2 underlined) and for amplification of the 377 bp region (377 bp region in bold, Primer 3 and Primer 4 underlined); amino acid sequences of the about 15 kDa and the about 31 kDa proteins are shown above the DNA sequences and are marked with arrows (small arrow about 15 kDa ORF 1, strong arrow about 31 kDa ORF 2);

Fig. 3. shows a DNA sequence of the 440 bp *M. tuberculosis* chromosomal region including the 380 bp region (in bold) used in PCR experiments and the amino acid sequence of the ORF 3 shown below the complementary DNA strand (< ORF 3);

Fig. 4 is an overview of the isolated lambda gt11-clone C9-2; 7.2 kb insert fragment, sequenced chromosomal regions and ORF 1, ORF 2 and ORF 3 marked with arrows;

Fig. 5 shows the amino acid sequence of the about 15 kDa protein (ORF 1);

Fig. 6 shows the amino acid sequence of the about 31 kDa protein (ORF 2);

Fig. 7 shows the amino acid sequence of the about 17 kDa protein;

Fig. 8 A shows SDS-PAGE of the insoluble pellet fraction (lane 1) and the purified about 15 kDa recombinant antigen (lane 2); lane A3 shows protein molecular weight standards (2.850 to 43.000 molecular weight range);

Fig 8 B shows SDS-PAGE of the purified about 15 kDa thioredoxin fusion protein (lane 1) and the two protein bands obtained after enterokinase cleavage (lane 1);

Fig. 9 shows a DNA sequence of *M. tuberculosis*;

Fig. 10 is a schematic drawing of the clone Mtub-Clara-Klon; the open reading frames of about 9 kDa (bp 3536 to bp 3829), 55 kDa (bp 2111 to bp 3829), 74 kDa (bp 1538 to bp 3829) and 77 kDa (bp 2698 to bp 2 on the complimentary strand) proteins are shown by arrows and the corresponding coding regions are numbered;

Fig. 11 A shows are southern hybridization with genomic DNA from different mycobacteria digested with PvuII (1: *M. tuberculosis* H37Rv; 2: *M. avium*; 3: *M. kanssasi*; 4: *M. necroti*; 5: *M. fortuitum*; 6: *M. phlei*; 7: *M. smegmatis*; 8: *M. vaccae*);

Fig. 11 B shows a finger-print obtained using the DNA (BamHI digest) of (1) *M. tuberculosi*s H37 RV, (2) *M. tuberculosis* H37 Ra, (3) *M. bovis* BCG, and (4) *M. tuberculosis* H37Rv digested with SalI;

Fig. 12 shows a finger-print with DNA from different *M. tuberculosis* clinical isolates (numbered 1 to 12) digested with PvuII restriction enzyme; the 4 kb Sal I fragment (Mtub-Klar-Klon) was used as probe;

Fig. 13 shows an amino acid sequence of the protein of about 74 kDa (molecular weight 74999, length 764)

Fig. 14 shows a glycine rich protein of about 77 kDa (molecular weight 77056, length 899);

Fig. 15 shows the amino acid sequence of the about 9 kDa proline rich protein (molecular weight 9356, length 98); and

Fig. 16 shows the proline rich protein of about 55 kDa (molecular weight 55982, length 573).

Modes for Carrying out the invention

We were interested in identifying and cloning novel DNA sequences from the genome of *Mycobacterium tuberculosis* for use in rapid and specific diagnosis of tuberculosis. Our strategy was to go for new repeated elements and insertion elements which are present only in *M.tuberculosis* or in the strains of *M. tuberculosis complex*.

Examples

The following examples further describe the isolation and sequencing of *M. tuberculosis*-DNA containing putative IS-element (Insertion Element) and repeat sequences, e.g., PGRS-elements (Polymorphic GC-Rich-Sequences) and the use of the as yet unreported DNA sequences for strain identification and diagnosis of tuberculosis.

Escherichia coli strains, phages and plasmids: The *Escherichia coli* K12 strain Y1090r - (Huynh et al., 1985) was used to propagate the λ gt11 library and the *E. coli* K12 strain GI724 (Invitrogen, Leek, The Netherlands) was the host for the production of the ca. 15kDa protein fused to thioredoxin.

The recombinant DNA library of *M. tuberculosis* genomic DNA in the λ gt11 expression vector was constructed by Young et al. (1985).

The plasmid vector pTrxFus (Invitrogen, Leek, The Netherlands) was used to make an in-frame fusion with thioredoxin as an amino-terminal fusion partner.

Mycobacterial strains and preparation of cell extracts: The mycobacterial strains used in this study are shown in Table 1 (Results and Discussion). All organisms

were grown on Loewenstein medium. For preparing cell extracts a loop of bacteria was suspended in 0.5 ml of 10 mM Tris/base, 1 mM EDTA (pH 7.4) followed by addition of 0.5 ml glass beads (150-212 microns, Sigma, Deisenhofen, Germany). The suspension was incubated at 80°C for 10 min followed by a 1 min treatment in a Mini-Bead Beater (Biospec Products).

DNA sequence analysis: Similarity comparisons were done using the BLAST program (Pearson and Lipman, 1988; NCBI computing facility).

All DNA manipulations were done according to standard procedures (see Maniatis et al. 1982).

DNA sequencing: DNA sequencing analysis was performed by the dideoxynucleotide-chain termination method using a PCR sequencing kit (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Warrington, Great Britain) on a 373A DNA Sequencer (Applied Biosystems, Warrington, Great Britain). DNA sequences were determined for both strands by primer walking.

1. Clone containing putative IS-Element

1.1 Isolation of the clone C9-2 containing a putative IS element:

In our attempt to isolate new mycobacterial insertion elements, a λ gt11 M. tuberculosis library was screened with oligodeoxyribonucleotide primers based on conserved regions of different insertion elements. The library was screened as described by Young and Davis (1985). Briefly, phage-infected cells of the strain E.coli Y1090r⁻ were plated in top agar on Luria-Bertani plates (7.0×10^6 PFU per 85 mm plate) and incubated for 6-8 h at 42°C. Nylon membranes (Biodyne B Transfer Membrane, 0.45 μ m, Pall, Portsmouth, England) were overlaid on plates. The filters were treated with 0.5 N NaOH, 1.5 M NaCl and the DNA was fixed via UV-crosslinking. Screening was performed using 3'-end labeled oligonucleotides of the sequence 5'-TGACGCGAGTGGGTGTGATTTCG-3' and 5'-GTGGTCGAGCCGTTGATGCCG-3' (Fig.2, PRIMER 1 and PRIMER 2). Digoxigenin-labeling of the oligodeoxyribonucleotide primers was carried out using a DIG Oligonucleotide 3'-End Labeling Kit (Boehringer Mannheim, Germany). Hybridization was done at 45°C in hybridization buffer (Boehringer Mannheim, Germany) overnight. Then the membranes were washed under stringent conditions for 5 min twice in 2 x SSC, 0.1% SDS and for 15 min twice at 37°C in 0.1 x SSC, 0.1% SDS. Chemiluminescent detection was carried out with the help of a DIG Luminescent Detection Kit

(Boehringer Mannheim, Germany). Plaques were purified by three rounds of plating to obtain single plaques. Phage DNA was isolated using a Nucleobond AX L50 Kit (Machery-Nagel, Düren, Germany) and restriction mapping of the selected clone was performed by standard procedures (Maniatis et al., 1982).

Several positive clones were obtained. Detailed analysis of one of the clones (C9-2) is presented here. The recombinant phage was mapped with the restriction endonucleases BamHI, EcoRI and KpnI (Fig. 1). EcoRI digestion revealed a 7.2 kb DNA insert fragment.

1.2 DNA sequencing of the cloned fragment:

Two *M. tuberculosis* chromosomal regions of 2253-bp and 440-bp of this fragment were sequenced (Fig.2 and Fig.3). DNA sequencing of the 2253-bp region revealed the presence of a putative insertion element between bp 401 and bp 1378 containing inverted repeats flanked by duplications of 4 base pairs. The cloned fragment reported here is novel and is located at a different position than the 2.1 kb PstI/EcoRI fragment reported by Mariani et al. (1993), because the DNA sequence of the adjoining regions on the left and the right ends of the putative IS-element were completely different in our clone C9-2 as compared to that reported by Mariani et al. (1993).

Fig.4 gives an overview of the 7.2 kb insert fragment and the sequenced chromosomal regions.

1.3 Novel Proteins coded by the cloned DNA:

During the molecular characterization of the clone, novel ORFs were identified. The complete ORF of the ca. 15kDa protein is located on the 2253-bp fragment coded by a 408-bp fragment, corresponding to a coding capacity of 136 amino acids. The ca. 15kDa protein (Fig.5) is a novel product showing limited homology in the N-terminus of a 34kDa ORF reported by Mariani et al. (1993). We also identified an ORF of about 31kDa (Fig. 2 and Fig. 6) coded by the cloned DNA (bp 515 till bp 1378). This 31kDa ORF did not show any homology in the N-terminus to any known sequence in the database. The C-terminus of the ca. 31kDa protein showed homology to a 34kDa ORF (Mariani et al., 1993). We have not used the DNA sequence showing homology to the sequence reported by Mariani et al. (1993) as far as the claims of this patent application are concerned. An ORF (ORF 3, Fig. 3 and Fig.7) on the complementary strand to the 3'-end of the insert fragment of the recombinant λ -clone C9-2 was identified, which had not been reported earlier. This sequence showed homology to a family of transcription regulators in microorganism. In addition, some homology was observed with a

putative two-component system *mtrA*-*mtrB* isolated from *M.tuberculosis* H37Rv (Via et al., 1996) and to PhoP of *Bacillus subtilis* (Lee and Hulett, 1992). Based on this data, the DNA sequence (440-bp fragment, Fig. 3) and the derived polypeptide might play a role in regulation of virulence in mycobacteria.

1.4 Cloning, expression and purification of the ca. 15kDa protein fused to thioredoxin

The λ gt11 clone C9-2 (Fig. 4) was used as template to amplify a PCR fragment of 951-bp (Fig. 2, sequence position 451-1378) including the ORF for the ca. 15kDa protein (Fig. 5) and cleavage sites for the restriction endonucleases *Sma*I and *Sal*I at the 5'- and 3'-ends. Amplification of the *Sma*I-*Sal*I mycobacterial DNA fragment for insertion into pTrxFus (Invitrogen, Leek, The Netherlands) was done using the oligonucleotide primers with the sequence 5'-TCTAGACATATGACGCGAGTGGGTGTGATTTCG-3' (PRIMER 7, forward) and 5'-CATATGGTTCGACCTAGGGCGTGTCTCCCAA-3' (PRIMER 8, reverse) corresponding to sequence positions 451-474 and 1378-1361 (Fig. 2). Composition of the reaction mix was the same as described above with 400 ng phage DNA as template. The probe was amplified in 30 cycles consisting of the same conditions as described. Cleavage sites were introduced by appropriate primers. After digestion with both restriction endonucleases the product was inserted in pTrxFus (Invitrogen, Leek, The Netherlands) to form the plasmid pCH3-8.

The *E. coli* strain GI724 was electroporated with the plasmid pCH3-8. Bacterial cultures (200 ml of Induction Medium (Invitrogen, Leek, The Netherlands) supplemented with 100 μ g/ml ampicillin) grown at 30°C were induced to synthesize the fusion protein by tryptophan addition (100 μ g/ml) and temperature shift to 37°C. Cells were collected after 4 hours (10 000 x g, 5 min, 4°C), resuspended in 4 ml Osmotic Shock Solution (Invitrogen, Leek, The Netherlands), broken by three rounds of alternate sonication on ice (10 sec.) and shock freezing in liquid nitrogen, and pelleted (10 000 x g, 15 min, 4°C). Most of the fusion protein accumulated in the form of inclusion bodies and only a small fraction was present as soluble protein inside the cells. The pellet containing the inclusion bodies was resuspended (denaturation) in 10 ml 6 M guanidine/HCl (pH 8.5), incubated for 2 hours at room temperature and pelleted again (10 000 x g, 30 min, 4°C). The recombinant fusion protein was refolded by dialysing against 50 mM Tris/HCl (pH 8.0). Anion exchange chromatography was done with the help of a BioCAD perfusion system (Perseptive Biosystems) on a Poros column HQ/M (Perseptive Biosystems).

Elution was performed using a linear NaCl gradient (0-1M). The fusion protein concentration was determined with the BioRad Protein Assay Kit (BioRad, Munich, Germany). Purity was assessed by densitometry (Molecular Dynamics, Software Image Quant) and analytical SDS-PAGE and coomassie staining.

The ca. 15kDa protein fused to thioredoxin was refolded as described above. Further purification of the ca. 15kDa protein fused to thioredoxin was carried out by anion exchange chromatography (Fig. 8, A lane 3 and B lane 1). After enterokinase cleavage of the purified ca. 15kDa protein fused to thioredoxin two protein bands were detectable on SDS-PAGE (Fig. 8, lane 2). By western blotting with a thioredoxin monoclonal antibody the lower 11kDa band was identified to be thioredoxin. The upper band corresponds to the ca. 15kDa recombinant protein of *M. tuberculosis*. This is the first report of expression and purification of the ca. 15kDa protein of *M. tuberculosis* in *E. coli*.

1.4. Species specific diagnosis of mycobacteria :

Deprotected and desalted Oligonucleotide primers were obtained from Gibco BRL (Eggenstein, Germany) or Eurogentec (Seraing, Belgium).

The oligodeoxyribonucleotide primers with the sequence 5'-GTCCATGTGCCGCCG CTG-3' (PRIMER 3, forward) and 5'-CTGCGCGGCTCCCGGCA-3' (PRIMER 4, reverse), specific for the DNA regions of the 2253-bp *M. tuberculosis* chromosomal region shown in Fig. 2 were used in PCR experiments to amplify a 377-bp fragment.

For amplification of a 380-bp fragment from the 440-bp chromosomal fragment, the oligodeoxyribonucleotide primers with the sequences 5'-CGAGGCTGAACGGCT TTG-3' (PRIMER 5, forward) and 5'-TCAACGTCCGCGGCAAGC-3' (PRIMER 6, reverse) corresponding to the DNA region shown in Fig. 3 were used. Amplifications were performed in 0.2 ml Micro Amp Reaction Tubes (Perkin Elmer, Norwalk, Connecticut, USA) in a final volume of 100 µl using a GeneAmp® PCR Kit (Perkin Elmer, Branchburg, New Jersey, USA). Reaction mixtures contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 200 µM dNTP, 0.1 µM Primer, 30-100 ng chromosomal DNA from mycobacterial cell extracts (Table 1) and 2.5 U AmpliTaq® DNA polymerase. All components of a PCR reaction except for the template are included in the Kit. The reactions were performed using the automated Thermal Cycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, Connecticut, USA). The samples were amplified by 40 cycles consisting of denaturation at 96°C for 2 min, annealing of the

primers at 25°C for 1 min and primer extension at 72°C for 3 min.

After amplification, 10 µl of each product was electrophoresed in a horizontal 1.5% agarose gel. Gels were precasted using a 1:10 000 dilution of SYBR Green I stock reagent (Eugene, Leiden, The Netherlands) in 10 mM Tris/HCl, 1 mM EDTA (pH 8.0).

For DNA sequencing the appropriate 377-bp and 380-bp PCR products from the mycobacterial cell extract samples (Table 1) were purified from an 1.5% agarose gel using a Gel Extraction Kit (QIAGEN, Hilden, Germany).

1.4.1. The 377-bp region:

The 377-bp region (Fig.2) of the isolated and sequenced 2253-bp *M. tuberculosis* chromosomal fragment and the 380-bp region (Fig.3) of the identified 440-bp chromosomal fragment were examined for their suitability for strain differentiation (Table 1). A PCR-product of the predicted size and a 100% DNA sequence homology in the 377-bp region was detected only in the members of the *M. tuberculosis* complex. No amplification product was obtained from other mycobacteria (Table 1). Therefore, the PCR primers of the 377-bp region are useful for the rapid discrimination of *M. tuberculosis* complex (*M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium africanum* and *Mycobacterium microti*) from other mycobacteria.

1.3.2. The 380-bp region:

A predominant amplification product of correct size of the 380-bp region was obtained from the chromosomal DNA samples of the *M. tuberculosis* complex including the vaccine strain *M. tuberculosis* BCG, the tuberculosis isolate Tub118 and the mycobacterial species *M. asiaticum*, *M. gastri*, *M. gordonae* and *M. kansasii*. Thus, this fragment can be used for the identification of above mycobacterial species, since no amplification product was obtained from other mycobacterial species (Table 1).

2. Clone containing PGRS Element

2.1. Cloning of DNA fragment containing PGRS elements:

We screened Lawrist cosmid library of *M. tuberculosis* DNA using a degenerate oligonucleotide of the sequence 5'-

C/GGCC/GGCC/GGGC/GACC/GGGC/GGGC/GGCCGGCTCC/GGG- 3' which was designed in such a way that it contained GC rich regions as well as it coded for a putative proline rich polypeptide. Colony hybridization using labelled oligonucleotide was performed using standard

procedures (Maniatis et al.1982). Filters were prehybridized and probed at 42°C overnight in a solution containing 6xSSC, 1 mM Sodium phosphate, 1mM EDTA, 0.05% skimmed milk, 0.5%SDS. Filters were washed twice in 2xSSC;0,3%SDS for 15 min at 65°C.

First screening yielded six positive clones which were rechecked by hybridization with the oligonucleotide. Three clones gave strong signal and restriction mapping of the clones showed identical restriction pattern. Further restriction mapping and Southern hybridization of one of the clones called identified an about 4kb Sall fragment that hybridized strongly to the oligonucleotide.

2.2. DNA sequencing of the cloned fragment: The ca. 4kb Sall fragment was subcloned in pUC19 and the clone was named Mtub-Clara-Klon. Entire insert was sequenced by primer walking method. The DNA sequence is presented in figure 9. There were unusual difficulties in obtaining the sequence of the recombinant clone because of the high GC rich content and due to the presence of unusual repeats.

2.3. Proteins coded by the cloned DNA:

We identified at least 4 ORF (open reading-frames) belonging to a ca. 9kDa, 55kDa, 74kDa and a 77kDa protein (Fig. 10). Interestingly, the amino acid sequence of the 9kDa, 55kDa, 74kDa and the 77kDa proteins didnot show strong homology to any sequences reported so far for Mycobacteria (Genbank and Swissprot Databases) . In addition, the 9kDa, 55kDa and the 74kDa proteins have an unusually high content proline, nevertheless, no strong homology with the known proline rich antigens (Laqueyrie et al. 1995; Infect.Immun.63,4003) of mycobacteria was observed. Unexpectedly, the amino acid sequence showed restricted homology to Mucin like proteins from eucaryotes. The 77kDa protein is highly rich in amino acid glycine and may be a cell wall protein of Mycobacterium tuberculosis. Such proteins have not been reported from M. tuberculosis.

2.3. DNA finger-printing:

The ca. 4kb Sall fragment was used to probe (Southern hybridization) genomic DNA of different mycobacteria digested by PvuII (Fig. 11). The results show that each strain showed a characteristic pattern making the differentiation of M. tuberculosis-Rv, M. tuberculosis-Ra, M. bovis and the M. tuberculosis Erdman strain. The ca. 4kb Sall fragment is also suitable for finger printing of clinical isolates, since hybridization of the probe to the genomic DNA of clinical isolates from tuberculosis patients also yielded strain specific finger print (Fig. 12). No hybridization to the genomic DNA of M. smegmatis, M. vaccae, M. avium, M. chelonie, M.

fortituum, *M. phlei* was observed.

REFERENCES

- Aminosante, M., Barnini, S., Corsini, V., Paone, G., Read Jr, C.A., Tartoni, P.L., Singh, M., Albera, C., Bisetti, A., Senesi, S., Campa, M. and Saltini, C.: Evaluation of a novel tuberculosis complex-specific 34 kDa protein in the serological diagnosis of tuberculosis. *Er. Respir. J.* **8** (1995) 2008-2014
- Barnes, P.F., Bloch, A.B., Davidson, P.T. and Snider Jr., D.E.: Tuberculosis in patients with human immunodeficiency virus infection. *New Engl. J. Med.* **324** (1991) 1644-1650
- de Wit, D., Maartens, G. and Steyn, L.: A comparative study of the polymerase chain reaction and conventional procedures for the diagnosis of tuberculous pleural effusion. *Tuber. Lung Dis.* **73** (1992) 262-267
- Glennon, M., Smith, T., Cormican, M., Noone, D., Barry, T., Maher, M., Dawson, M., Gilmarin, J.J. and Gannon, F.: The ribosomal intergenic spacer region: a target for the PCR based diagnosis of tuberculosis. *Tuber. Lung Dis.* **75** (1994) 353-360
- Grybowski, S.: Tuberculosis in the third world. *Thorax* **46** (1991) 689-691
- Huynh, T.V., Young, R.A. and Davis, R.W.: In: *DNA Cloning*, Glover, D.M., ed., Vol. 1, IRL, Oxford, England (1985), 56-110
- Kochi, A.: The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle* **72** (1991) 1-6
- Lee, J.W. and Hulett, F.M.: Nucleotide sequence of the *phoP* gene encoding PhoP, the response regulator of the phosphate regulon of *Bacillus subtilis*. *Nucleic Acids Res.* **20** (1992) 5848
- Maniatis, T., Fritsch, E.F. and Sambrook: *Molecular cloning: a laboratory manual*. (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Mariani, F., Piccolilla, E., Colizzi, V., Rappuoli, R. and Gross, R.: Characterization of an IS-like element from *Mycobacterium tuberculosis*. *J. Gen. Microbiol.* **139** (1993), 1767-1772
- Miller, S.I.: PhoP/PhoQ: macrophage-specific modulators of *Salmonella* virulence? *Mol. Microbiol.* **5** (1991), 2073-2078
- Miller, J.F., Mekalanos, J.J. and Falkow, S.: Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **243** (1989), 916-922
- Narita, M.: Polymerase chain reaction for diagnosis of infectious disease. *Acta Paediatr.*

Jpn. 35 (1993), 89-97

Pearson, W.R., Lipman, D.J.: Improved tools for biological sequence comparison. Proc. Nat. Acad. Sci. 85 (1988), 2444-2448

Selwyn, A.P., Hartel, D., Lewis, V.A., Schoenbaum, E.E., Vermund, S.H., Klein, R.S., Walker, A.T. and Friedland, G.H.: A prospective study of the risk of tuberculosis among intravenous drug users with the human immunodeficiency virus infection. New Engl. J. Med. 320 (1989) 545-550

Shankar, P., Manjunath, N. and Mohan, K.K.: Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. Lancet, 337 (1991), 5-7.

Via, L.E., Curcic, R., Mudd, M. H., Dhandayuthapani, S., Ulmer, R.J., and Deretic, V.: Elements of signal transduction in Mycobacterium tuberculosis: in vitro phosphorylation and in vivo expression of the response regulator MtrA. J. Bacteriol. 178 (1996) 3314-3321

Vismara, D., Filippone Mezzopreti, M., Gilardini, M., Del Porto, P., Lombardi, G., Piccolella, E., Damiani, G., Rappuoli, R. and Colizzi, V.: Identification of a 35-Kilodalton Mycobacterium tuberculosis protein containing B- and T-cell epitopes. Infect. Immun. 58 (1990) 245-251

Wilkins, E.G.L.: The serodiagnosis of tuberculosis. In: Clinical Tuberculosis. Davies P.D.O. (ed.) (1994) Chapman & Hall, London.

Young, R.A., Bloom, R.B., Grosskinsky, C.M., Ivanyi, J., Thomas, D. and Davis R.W.: Dissection of Mycobacterium tuberculosis antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA 82 (1985) 2583-2587.

Young, R.A. and Davis, R.W. (1985) Constructing and screening cDNA library in λ gt10 and λ gt11, p. 49-78. In: Genetic engineering: principles and methods, J.K. Setlow and A. Hollaender (ed.), vol. 7. Plenum Publishing Corp., New York.

Table 1 Distribution of the 377-bp sequence and the 380-bp sequence in different mycobacteria

Species		Presence of the 377-bp fragment	Presence of the 380-bp fragment
<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> H37Rv	+	+
	<i>M. tuberculosis</i> H37Ra	+	+
	<i>M. bovis</i>	+	+
	<i>M. africanum</i>	+	+
	<i>M. microti</i>	+	+
vaccine strain	<i>M. bovis</i> BCG	+	+

		Species	Presence of the 377-bp fragment	Presence of the 380-bp fragment
clinical tuberculosis isolate	Tub 118		+	+
		<i>M. asiaticum</i>	-	+
		<i>M. avium</i>	-	-
		<i>M. chelonae</i>	-	-
		<i>M. flavescens</i>	-	-
		<i>M. fortuitum</i>	-	-
		<i>M. parafortuitum</i>	-	-
		<i>M. gastri</i>	-	+
		<i>M. gordonae</i>	-	+
		<i>M. intracellulare</i>	-	-
		<i>M. kansasii</i>	-	+
		<i>M. lufu</i>	-	-
		<i>M. marinum</i>	-	-
		<i>M. nonchromogenicum</i>	-	-

Species	Presence of the 377-bp fragment	Presence of the 380-bp fragment
<i>M. pergrinum</i>	-	-
<i>M. phlei</i>	-	-
<i>M. scrofulaceum</i>	-	-
<i>M. simiae</i>	-	-
<i>M. smegmatis</i>	-	-
<i>M. terrae</i>	-	-
<i>M. ulcerus</i>	-	-
<i>M. vaccae</i>	-	-
<i>M. xenopi</i>	-	-
<i>M. thermoresistibile</i>	-	-
<i>M. triviale</i>	-	-
none		
<i>Nocardia asteroides</i>	-	-
<i>Rodococcus equi</i>	-	-
tuberculosis strains		

Claims**1. (I) DNA**

- (a) having sequence (I) according to figure 9, wherein optionally one or more codons can be replaced by codons coding for the same amino acid(s),
- (b) having a sequence complementary to said of (a),
- (c) being single-stranded, wherein its strand is hybridizable with that of the DNA according to (a) or (b),
- (d) being double-stranded, the sequences of its single strands being defined as in (a) and (b), respectively,
- (e) being double-stranded, its single strands being hybridizable with those of the DNA according to (d), or
- (f) being a subsequence of the sequences according to (a) to (e); or

(II) DNA

- (a) having sequence (VI) according to figure 2, wherein optionally one or more codons can be replaced by codons coding for the same amino acid(s),
- (b) having a sequence complementary to said of (a),
- (c) being single-stranded, wherein its strand is hybridizable with that of the DNA according to (a) or (b),
- (d) being double-stranded, the sequences of its single strands being defined as in (a) and (b), respectively,
- (e) being double-stranded, its single strands being hybridizable with those of the DNA according to (d), or
- (f) being a subsequence of the sequences according to (a) to (e); or

(III) DNA

- (a) having sequence (IX) according to figure 3, wherein optionally one or more codons can be replaced by codons coding for the same amino acid(s),

- (b) having a sequence complementary to said of (a),
- (c) being single-stranded, wherein its strand is hybridizable with that of the DNA according to (a) or (b),
- (d) being double-stranded, the sequences of its single strands being defined as in (a) and (b), respectively,
- (e) being double-stranded, its single strands being hybridizable with those of the DNA according to (d), or
- (f) being a subsequence of the sequences according to (a) to (e).

2. A DNA according to claim 1 (I) (c), (I) (e), (II) (c), (II) (e), (III) (c) or (III) (e), its single strands being hybridizable at a temperature of at least 25 °C and at a concentration of NaCl of 1 M.

3. RNA being a transcript of a DNA according to claim 1 or 2.

4. Protein being encoded by a DNA according to claim 1 or 2.

5. Protein having the amino acid sequence (II) according to figure 13.

6. An about 74 kDa protein according to claim 4 or 5.

7. Protein having the amino acid sequence (III) according to figure 14.

8. An about 77 kDa protein according to claim 4 or 7.

9. Protein having the amino acid sequence (IV) according to figure 15.

10. An about 9 kDa protein according to claim 4 or 9.

11. Protein having the amino acid sequence (V) according to figure 16.
12. An about 55 kDa protein according to claim 4 or 11.
13. Protein having the amino acid sequence (VII) according to figure 5.
14. An about 15 kDa protein according to claim 4 or 13.
15. Protein having the amino acid sequence (VIII) according to figure 6.
16. An about 31 kDa protein according to claim 4 or 15.
17. Protein having the amino acid sequence (X) according to figure 7.
18. An about 17 kDa protein according to claim 4 or 17.
19. A protein according to any of claims 4 to 18, wherein the protein is a recombinant protein, especially a protein produced by means of a bacterial strain, a yeast strain, a fungal strain or a cell line of a higher eucaryote.
20. A protein being encoded by a DNA sequence according to claim 1 or 2 and which can be recovered by a method comprising the following steps:
 - (i) subjecting proteins encoded by said DNA sequence to a usual test for diagnosis of tuberculosis,
 - (ii) selecting a protein showing an inhibitory effect and
 - (iii) isolating and recovering said protein.
21. DNA according to claim 1 or 2, RNA according to claim 3 or protein according to any of claims 4 to 20 which can be used for

(i) diagnosis of tuberculosis in humans and animals and/or
(ii) diagnosis of other mycobacterial infections in humans or
animals,
each especially by means of samples taken from humans or
animals.

22. Use of a DNA according to any of claims 1, 2 or 21 for the
identification of mycobacteria in media samples.

23. Use according to claim 22, comprising the steps of
(i) isolating the mycobacterium,
(ii) preparing crude or purified genomic DNA,
(iii) hybridizing it to a DNA according to claim 1 or 2 and
(iv) detecting the fragment pattern using conventional methods
such as a radioactivity assay, chemiluminiscence or
fluorescence.

24. Use according to any of claims 22 to 23, wherein as samples
clinical samples are used.

25. Use of a DNA according to any of claims 1 to 2 or of a
protein according to any of claims 4 to 20 for
(i) epidemeological purposes and/or
(ii) vaccination follow-up
for humans or animals suffering from mycobacterial infections,
especially tuberculosis.

26. Use of a DNA according to any of claims 1 to 2 or of a
protein according to any of claims 4 to 20 for the development
of drugs useful for combating mycobacterial infections of humans
or animals, especially tuberculosis, especially for testing and
recovering of substances inhibiting mycobacterial infections in
humans and animals, especially tuberculosis.

1/23

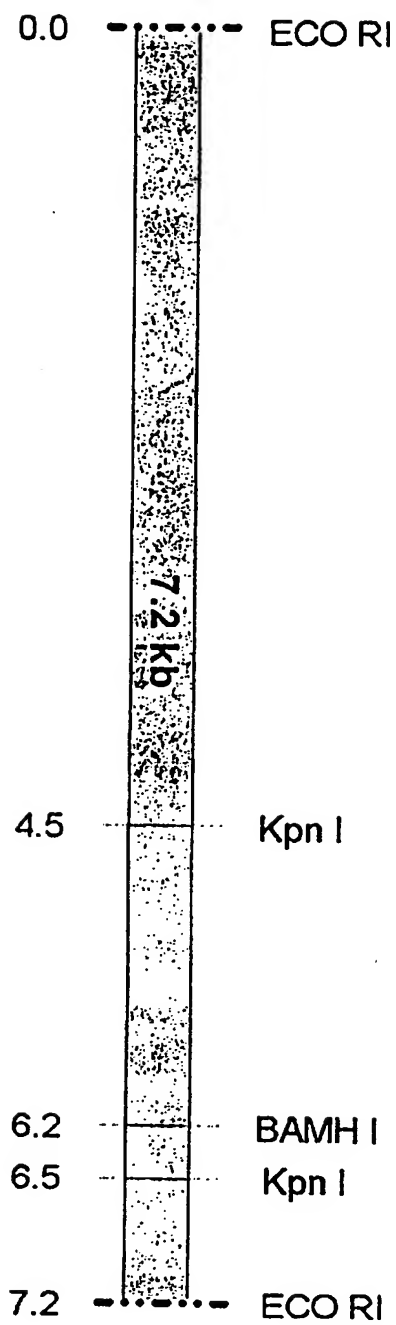


Fig. 1 Restriction endonuclease map of the 7.2 kb *M. tuberculosis* chromosomal region

Fig. 2 - 1/4

1 CGACGCCGT GTGATCGGCC TCGTGTGCGA GGAATCTGCG GAAGCACCGT CGACGGGCAC CGGACCGAAC GCGGCACCCT TCTATCGCG CATCCCGTCG
 GCTGCCGGCA CACTAGCCGG AGCACCAGCT CCTGAGACGC CTTCGTGGCA GCTGCCCGTG GCCTGGCTTG CGCGTGGA AGATAGCGCC GTAGGCGACG
 101 AGCGAAGTGA TCCGAGCACT CGACGAATC GACTTCGGG GCATCGTGA GATGGACACC TTCCCATAGG CACAACCCGG GACGIATCAA CAGGCACCAA
 TCGCTTCACT AGGCTCGTGA GCTGCTTGA CTGAAGCCG CGTAGCACCT CTACCTGTGG AAGGTATCC GTGTTGGGCC CTGCATAGT GTGCGTGGT
 201 ACCCTTCAAT CCGGTGGTAG TGGGTGAGT TGATTTTCG CGAATCTGAC GCGGCGTAG CTCGCTGTGT CTAGGCAAGG CATGGTGTGG CTAGGCAAGG
 TGGGAAGTTA GGCCACCATC ACCCAGTCAA ACTAAAGCG GCTTGAGCTG CGCCGATCC GAGCGACACA GATCCGTCCC GTACCACACC GATCCGTTC
 301 CTAGGAATGG CTTGGCGTGG CAAGGCAAG TTTGAGCCAG CGGGCCGCG CCATAGGAGA TCGGCCGAAC AGGCCAAGT ACTACCGCTG CAGCCCCGCG
 GATCCTTACC GAACCGCACC GTTCCGTTCC AACTCGGTC GCCCGCGCTC GGTATCTCT ACAGCGCTTG TCCGGTTCA TGATGGCGAC GTCGGGCGCG
 ORF 1 → ValThrArgVal GlyValIle SerAspGlu PheTrpAlaVal ValGluPr
 PRIMER 1
 401 CTAGGGCGTG TCTCCCAAAT TTTTAGGTAC TGGCCAGCGA GGATTGGCCG GTAGCGGAG TGGTGTGAT TTGGACGAG TTCTGGGCGG TGCTCGAGCC
 GATCCCGCAC AGAGGGTTA AAAATCCATG ACCGCTCGCT CCTAACCGGC CACTGGCTC ACCACACTA AAGCTGCTC AAGACCCGGC ACCAGCTCGG
 PRIMER 2
 ORF 2 → MetArg AlaSerPro AlaAspGly LeuAlaIleThr GlyLeuSer TrpLysGly SerArgGlyGly SerValArg GluValArgG1
 ORF 1 oLeuMetPro SerHisGluGly LysProGly ArgArgPhe SerAspHis ArgLeuIleLeu GluGlyIle AlaTrpArgPhe ArgThrGly SerProTrp
 501 GTTGATGCCG TCGCATGAGG GCAAGCCCCG CAGACGGTTT AGCGATCACC GGCTTATCCT GGAAGGGATC GCGTGGCGGT TCCGTACGGG AAGTCCGTGG
 CAACTACGGC AGCGTACTCC CGTTCGGGCC GTCTGCCAAA TCGCTAGTGG CCGAATAGGA CCTTCCCTAG CGCACCGCCA AGGCATGCCC TTCAGGCACC
 ORF 2 YGlyThrCys ProLeuSer SerGlyArg GlyLysArgCys GlySerAla IleThrValGly ArgTrpMet ValProAla ThrArgCys SerProThrLeu
 ORF 1 ArgAspLeuPro AlaGluPhe GlyProTrp GlnThrValTrp LysArgHis HisArgTrp SerLeuAspGly ThrCysAsp GluValPhe AlaHisValA
 KpnI
 601 CGGGACCTGC CCGCTGAGTT CGGGCCGTGG CAAACGGTGT GGAAGCGCCA TCACCGTGG TCGCTGGATG GTACCTCGGA CGAGGTGTTT GCCACGTTG
 GCCCTGGACG GCGGACTCAA GCCCGGCACC GTTTCACACA CCTTCGGGT AGTGGAACC AGCGACTAC CATGACGCT GCTCCACAAG CGGTGCAAC

Fig. 2 - 2/4

ORF 2 ProArgCys SerGlyTyr ThrLeuArgTyr ProArgIle SerArgSerCys CysArgTyr IleProArg ThrCysGly HisThrSerIle ArgArgAlaP

ORF 1 laAlaValPhe GlyValAsp AlaGluValAla GluAspIle GluLysLeu LeuSerValAsp SerThrAsn ValArgAla HisGlnHisSer AlaGlyAl

701 CCGCGGTGTT CCGGTGGAC GCTGAGTGG CCGAGGATAT CGAGAAGCTG CTGTGGTGG ATTCCACGAA CGTGGGGCA CACAGCATT CCGCGGGCGC

GGCGCACAA GCCCACCTG CGACTCCACC GGCTCCTATA GCTCTTCGAC GACAGCCACC TAAGGTGCTT GCACGCCCT GTGGTCGTAA GCCGCCCGCG

ORF 2 roAlaArg ThrArgSerPro GlnGlyAla LeuSerAsp TyrLysLysSer AlaAspGlu ProAspAsp HisAlaIleGly ArgSerArg GlyGlyLeuTh

ORF 1 aCysSerAsp ThrLeuAlaThr GlyGlyThr ValGlyLeu GlnGluIleA rgArg*** ←

801 CTGCTGGAC ACGCTGCGCA CAGGGGSCAC TGTGGGATTA CAAGAAATCC GCCGATGAC CCGACGATCA TCGGATCGGC CGCTCGCGG GCGGGCTGAC

GACGAGCCTG TCGAGCGGT GTCCCCCGTG ACAGCCTAAT GTTCTTTAGG CGGCTACTTG GGCTGCTAGT ACGTAGCCG GCGAGCGCGC GCGCCGACTG

ORF 2 rThrLysIle HisAlaLeu ThrAspGln ArgGluAlaPro ValArgIle ArgLeuThr AlaGlyGlnAla GlyAspAsn ProGlnLeu LeuProLeuLeu

BamHI

901 CACCAAGATC CATGCCCTGA CCGATCAGCG CGAGCCCCCG GTGCGGATCC GGTGACCGC AGGCCAGGCC GGCACAPAC CGCAACTGCT GCCCCTGCTC

GTGGTTCTAG GTACGGGACT GGCTAGTCGC GCTTCGGGC CACGCCTAGG CCAACTGGCG TCCGTCCGG CGCTGTTGG GCGTTGACGA CCGGGACGAG

ORF 2 AspAspTyr ArgHisAla IleThrGluTyr AlaLeuGly SerThrAspPhe ArgLeuLeu AlaLeuLys AlaTyrSer HisProSerThr ArgAlaAlaL

1001 GACGACTATC GCCATGCCAT CACCGAATAC GCCCTGGCA GCACGGATTT CCGCTTACTC GCGACAAGG CCTACTCACA CCCAAGTACC CGTCCCGCAT

CTGCTGATAG CGGTACGGTA GTGGCTTATG CCGGACCCGT CGTGCTTAA GCGAATGAG CGGCTGTTCC GGATGAGTGT GGGTTCATGG GCACGGCGTA

ORF 2 euArgSer LysLysIleLys HisThrIle ProGluArg GlnAspGlnIle AspArgArg LysAlaLys GlySerAlaGly GlyArgPro ProAlaPheAs

1101 TACGGTCTAA GAAGATCAAG CACACCTCC CCGAACGCCA AGATCAGATC GACCGCGCA AGGCCAAGG GTCTGCCGC GGCAGGCCAC CAGCATTCGA

ATGCCAGATT CTTCTAGTTC GTGTGGTAGG GGCTTGGGT TCTAGTCTAG CTGGCCGCGT TCCGGTTCC CAGACGGCGC CCGCCCGGTG GTCGTAAGCT

ORF 2 paAlaAlaLeu TyrGlyLeu ArgAsnThr ValGluArgGly PheHisArgLeu LysGln TrpArgGlyIle AlaThrArg TyrAspLys TyrAlaLeuThr

1201 GCGCGGCTC TACGGGCTAC GCACACCCGT CGAACGGCGC TTCCATCCAC TCAAGCAGTG GCGCGCATC GCAACCGCT ACGACAAATA CGCCCTGACC

GCGCGCGAG ATGCCCGATG CGTTGTGCA GCTTGGCCG AAGTAGCTG AGTTCGTCAC CGGCGCGTAG CGTTGGCGA TGCTGTTAT GCGGGACTGG

ORF 2 TyrLeuGly GlyValLeu LeuAlaCysAla ValIleHis AlaArgValGlyThrProLys LeuGlyAsp ThrPro*** ←

1301 TACCTCGCG GCGTCTGCT GCGCTCGCC GTCAATCCACG CCGAGTGGG AACTCCGAA TTGGGAGACA CGCCTAGTC GGGATACCTG CCGCACCGGT

ATGGAGCGC GCGAGGACGA CCGGACGCG CAGTAGGTG GGGCTCACCC TTGAGGCTTT AACCTCTGT GCGGATCAG CCTATGGAC GCGGTGCGCA

Fig. 2 - 3/4

1401	CCAGCTGGCT GTCCGTGACG GCGTGGGG TGCGCTGTC AAGGACGTG GGTTCGGCA AGATTGATG CCGAGGTTG TGCTCACTG CGACCGGAC GGTCGACCGA CAGGCACTGC CGCAGCGCC ACGGACACG TTCTGACG CCAACGGCGT TCGTAACATAC GGGTCCAAC ACGAGTTGAC GCTGGCCCTG
1501	GATCCGGGC AACGTGGCA CAACGTCGAG GCGATCCAGC AGGCGTTGC TCGGTTGCA GCACTGCCG ACCAAGCGT CCTACCCACG TTCCGGGCTT CTAGGGCCCG TTGCACCCGT GTTGCAGTC CGTAGGTCG TCCGGAACG AGCCAAGCGT CGTGGACGGC TGGTCCGA GGATGGGTGC AAGGCGGAA
1601	TCGACCTACC TCGTGTTCGA CGCATTGATC GCCACGGTG ATGTCACGA CCGCACTGG <u>GGGTCCATG TGCCGGCGT GGAGACAAG TATGTGGACG</u> AGCTGATGG AGCACAAGCT GCGTA-CTAG CGGGTGCCAC TAGCAGTGT GCGTTGACC CCGCAGGTAC ACGCGGGA CTTCTGTTTC ATACACCTGC
1701	CGCTGTCCC ATCGTTCGAC CACCGGCCA GCGTCGGGT CACGCTGACC GACCAGCCC CCGCTCAGCA TTTGCACGAC GGGTGAGCTT CCCTCTTTGT CGCACACGGG TAGCAAGCTG GTGCGCCGT CCGAGCCAA GTGCACTGG GCGGAGTCGT AACGTGCTG CCACTCGAA GGGGAGAACA
1801	TGTGAGACA TCGGTAGCC GCGCAACTC GCTGGCGAGC TTTTCTGCCA CGTCTTCGC GAGTAAACGA TCGCGCTCG GTGCGCGCG CTTGAAGACG ACACTCTGT AGCGCATCG CCGGTTGAG CGACCGCTG AAAGACGCT GCAGAAGCG CTCATTGCT AGCGGAGGC CAGCGCGGC GAACTTCTCG
1901	CAGTCCGCT TCTGGAAGA ATGTTTGAC CAATCGTGG GGTGTTGCC CTGGTCTCC AGCGCGCAA CAGACCACTC GTCAACGCAA ACGACCGCC GTGACGGGA AGACGCTTCT TACAACTCG GTTAGCACG CCACAACGG GACCCAGAG TCCGGCGTT GTCTGTCAG CAGTTGCTT TGCTGGCCG
2001	AGGAATCGG CACGAACCTA TTGTGCGGG AGCGCGCAG ACGCGTATC GATCAGCGG CCAACGCCCC CATCTGGATG CCGACAGTGC TGGACAGTGG TCCTTAGCCC GTGCTTGAGT AACACGGCC <u>TGGCGGTC</u> TGCCGCATAG CTAGTCGCG GGTTCGGGG GTAGACCTAC GGCTGTCAG ACCTGTCACC
2101	CTTCGACCG GCATATTGC PATTAGAAC GACTCTTTG AAGATTACCG GCACTAGCAA ACSCCAGCAC CCGTAGCGC GGATTGTCCG CTGCTCTGC GAAGCTGGC CGTATACCG TTAATCTTG CTGAGAAAGC TTCTAATGGC CGTATCGT TCGGTCGTG GGCACTCGCG CCAACAGGC GACGAGACG
2201	CAATCGGCT GTGAGGCTT GACGCGAAT GTACGCTTG GACCGATCC AGTGCACCAA CCTACCGC ACACGCCAGG TCGAAAGAG CCGTCATTG GTTAGCCCGA CACTCCGAA CTGCGCTTA CATCGGAAC CTGGCTAGG TCACGTGGT GGGATGGCG TGTGCGTCC ACGTTTCTC GGCAGTAAGC
2301	CCTAGAATC GCGGCGCG CGTCTCATTT GTGTGCCGA GTGTGCTAG CCGGGCACCA GATCACACCG CCATGTACG CGCCAAGACC TTGTAAAGGA GGATCTTAG CGCGCGCG GCAGAGTAA CACACGGCTT CACAGGATG GCGCGTGGT CTAGTGTGG GGTACAGTGC GCGTCTCTG AACATTCCT
2401	TGCTGACGTA AACGTTAAA AACCGTAAAC GTCGGGCTC CCATGCCCTT CCGTGGGCT TGTGGAGGT TGGCCGGTTA GCGTTACGCC AATCCACAGG ACGACTGCAT TTGCAATTT TTGGCATTTG CAGCCCGCAG GGTACGGAA GCCAGGCTG ACACCTCCGA ACCGGCCAAT CGCAATGCGG TTAGGTGTCC

PRIMER 3

PRIMER 4

Fig. 2 - 4/4

```

2501 CAGCCGTACG CAAACACACA GGGTGGCCC GGGTGCTAG CGCCGGCAG GTTTCACACA CAGGGAGCA ATGGATGTCA TPCCTCAAGA CAGTGCCTGA
    GTGGCATGC GTTGTGTGT CCCAGCCGG CCCACGATC GCGGCGCGT CAAAGTGTGT GTTCCCTCGT TACCTACAGT AAGGAGTTCT GTCAGGACT

2601 AGAGTTGACG GCTGGGCGG CGCAACTCGG GACCATCGGT GCTGCGATGG CAG
    TCTCAACTGC CGACGCGGC GCGTTCAGCC CTGCTAGCCA CGACGCTACC GTC

```

Fig. 2 2253-bp *M. tuberculosis* chromosomal region including BamHI, EcoRI and KpnI restriction sites and oligonucleotides for screening the λ gt11 *M. tuberculosis* library (PRIMER 1 and PRIMER 2 underlined) and for amplification of the 377-bp region (377-bp region in bold, PRIMER 3 and PRIMER 4 underlined). Amino acid sequences of the ca. 15kDa and the ca. 31kDa proteins are shown above the DNA sequences and are marked with arrows (\rightarrow ca. 15kDa ORF 1, \rightarrow ca. 31kDa ORF 2).

PRIMER 5

1 CGAGGCTGAA CGGCTTGTG A⁷CTACTGCT CGGGGGCCGA GGTGAGACCG GTGACCGGT CCATCAGCGA ATCGGGGCG GTGAGAACAA GGGTGGGTGT
 GCTCGACTT GCGGAAACAG TACATCAGCA GCGGGGGCT CCACTCTGCG CACTGGGCA GGTAGTGGCT TAGGGGGCG CACTCTTGT CCGACGACAA

→ LeuSerPhe ProLysThrMet TyrAspAsp AlaGlySer ThrLeuGlyThr ValArg AspMetValSer AspArgAlaThr LeuPheLeu ThrProThr

101 GTAGACCTCG GATTCTCGGA CCGGTGGCAG GATTTCACAT CCGGACGCAI GATGTGAGG ACCACACAT GGGGGCGGAC CTGTGTGCAAC
 CATCTGACC CTAGAGGCT GGGCAGGCTC CTAAAGTTG GCGAGGTGTA GCGCTTGTA CTACAGCTCC TGGTGTGTA GCGCGGCTG CAACAGCTTG
 TyrValAsp SerGluArgVal ArgArgLeu IleGluLeuGly AspValAsp ProLeuMet IleAspLeuVal LeuValAsp ProGlyVal LysAspPheL

201 TTGCTATCG CCTCTTGGC GTGCTGGCG ACTTCCACAT CCGAGCTTC GTAGTCAGC GGAATCTGA CCAATCTGT CAGGCTGTGT TGGTCAATGA
 AACCATACC GCGAACGCG CAGCACCGC TGAAGCTGA GGTGGGAG CATCACGTGC CCGTAGACT GGTCTAACCA GTGCGGACCA AGCAGTAGCT
 ysAlaIleAla GluGluGly AspHisAlaVal GluValAsp TrpGlyGlu TyrHisLeuAla MetLysVal LeuAsnThr LeuAlaProGlu AspAspVa

301 CCACACAC CCGGATGGT GATTCATCG CCGCATGAT CCGTGGCAGC TCGCCACAGA TGGTTGCGG GCGACGTGA CTGGGGGTGT ACCCGGACAT
 GCTTGTGNG GCGCTAGCCA CTAGGTAGGC GCGCTACTTA GGCACGGTGG AGGGGTCTT ACCEAAAGGC GCGTGGAACT GACGCGGACA TGGGGCTGTA
 IleuLeuVal ArgIleProSer GlyAspAla ArgHisIle ArgProLeuGln GlyLeuPhe AlaGlnArg ProArgGlnSer ArgThrTyr GlySerMet

PRIMER 6

401 GGTGCTCATG CTCGCGTATC CTTCAATTC TGTGCAAGCG
 GCAGCAGTAC GAGGGCATAG GAGAGTTAAG ACACGTTCCG

ThrThrMetSer GlyTyrGly ArgLeuGlu ThrCysAla ← ORF 3

Fig. 3 DNA sequence of the 440-bp *M. tuberculosis* chromosomal region including the 380-bp region (in bold) used in PCR experiments and the amino acid sequence of the ORF 3 shown below the complementary DNA strand (← ORF 3).

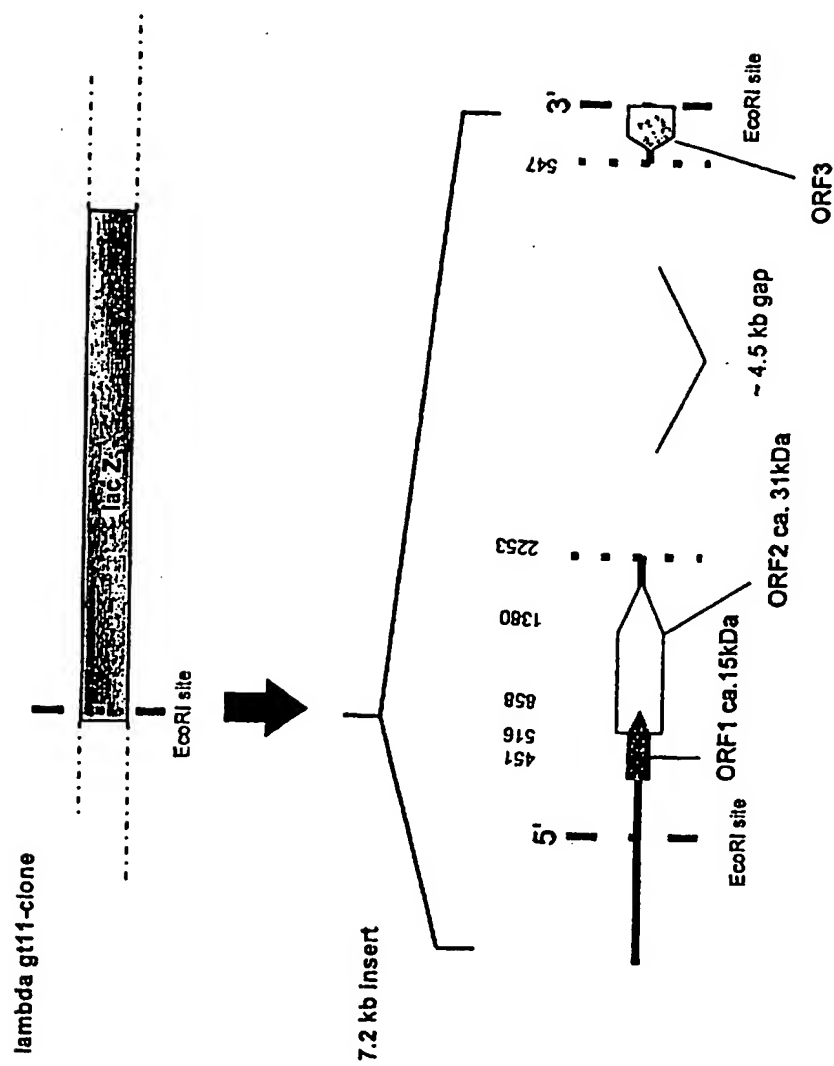


Fig. 4 Overview of the isolated λ gt11-clone C9-2: 7.2 kb insert fragment, sequenced chromosomal regions () and ORF1, ORF2 and ORF3 marked with arrows.

Val Thr Arg Val Gly Val Ile Ser Asp Glu Phe Trp Ala Val Val Glu Pro Leu Met Pro Ser His Glu Gly
Lys Pro Gly Arg Arg Phe Ser Asp His Arg Leu Ile Leu Glu Gly Ile Ala Trp Arg Phe Arg Thr Gly Ser
Pro Trp Arg Asp Leu Pro Ala Glu Phe Gly Pro Trp Gln Thr Val Trp Lys Arg His His Arg Trp Ser Leu
Asp Gly Thr Cys Asp Glu Val Phe Ala His Val Ala Ala Val Phe Gly Val Asp Ala Glu Val Ala Glu Asp
Ile Glu Lys Leu Ser Val Asp Ser Thr Asn Val Arg Ala His Gln His Ser Ala Gly Ala Cys Ser Asp
Thr Leu Ala Thr Gly Gly Thr Val Gly Leu Gln Glu Ile Arg Arg ***

Fig. 5 Amino acid sequence of the ca. 15kDa protein (ORF 1)

9/23

Met Arg Ala Ser Pro Ala Asp Gly Leu Ala Ile Thr Gly Leu Ser Trp Lys Gly Ser Arg Gly Gly Ser Val Arg Glu Val
 Arg Gly Gly Thr Cys Pro Leu Ser Ser Gly Arg Gly Lys Arg Cys Gly Ser Ala Ile Thr Val Gly Arg Trp Met Val Pro
 Ala Thr Arg Cys Ser Pro Thr Leu Pro Arg Cys Ser Gly Trp Thr Leu Arg Trp Pro Arg Ile Ser Arg Ser Cys Cys Arg
 Trp Ile Pro Arg Thr Cys Gly His Thr Ser Ile Arg Arg Ala Pro Ala Arg Thr Arg Ser Pro Gln Gly Ala Leu Ser Asp
 Tyr Lys Lys Ser Ala Asp Glu Pro Asp Asp His Ala Ile Gly Arg Ser Arg Gly Gly Leu Thr Thr Lys Ile His Ala Leu
 Thr Asp Gln Arg Glu Ala Pro Val Arg Ile Arg Leu Thr Ala Gly Gln Ala Gly Asp Asn Pro Gln Leu Leu Pro Leu Leu
 Asp Asp Tyr Arg His Ala Ile Thr Glu Tyr Ala Leu Gly Ser Thr Asp Phe Arg Leu Leu Ala Leu Lys Ala Tyr Ser His
 Pro Ser Thr Arg Ala Ala Leu Arg Ser Lys Lys Ile Lys His Thr Thr Ile Pro Glu Arg Gln Asp Gln Ile Asp Arg Arg Lys
 Ala Lys Gly Ser Ala Gly Gly Arg Pro Pro Ala Phe Asp Ala Ala Leu Tyr Gly Leu Arg Asn Thr Val Glu Arg Gly Phe
 His Arg Leu Lys Gln Trp Arg Gly Ile Ala Thr Arg Tyr Asp Lys Tyr Ala Leu Thr Tyr Leu Gly Gly Val Leu Leu Ala
 Cys Ala Val Ile His Ala Arg Val Gly Thr Pro Lys Leu Gly Asp Thr Pro ***

Fig. 6 Amino acid sequence of the ca. 31kDa protein (ORF 2)

10/23

Ala Cys Thr Glu Leu Arg Gly Tyr Gly Ser Met Thr Thr Met Ser Gly Tyr Thr Arg Ser Gln Arg Pro Arg
Gln Ala Phe Leu Gly Gln Leu Pro Arg Ile His Arg Ala Asp Gly Ser Pro Ile Arg Val Leu Leu Val Asp
Asp Glu Pro Ala Leu Thr Asn Leu Val Lys Met Ala Leu His Tyr Glu Gly Trp Asp Val Glu Val Ala His
Asp Gly Gln Glu Ala Ile Ala Lys Phe Asp Lys Val Gly Pro Asp Val Leu Val Leu Asp Ile Met Leu Pro
Asp Val Asp Gly Leu Glu Ile Leu Arg Arg Val Arg Glu Ser Asp Val Tyr Thr Pro Thr Leu Phe Leu Thr
Ala Arg Asp Ser Val Met Asp Arg Val Thr Gly Leu Thr Ser Gly Ala Asp Tyr Met Thr Lys Pro Phe
Ser Leu

Fig. 7 Amino acid sequence of the ca. 17kDa protein

11/23

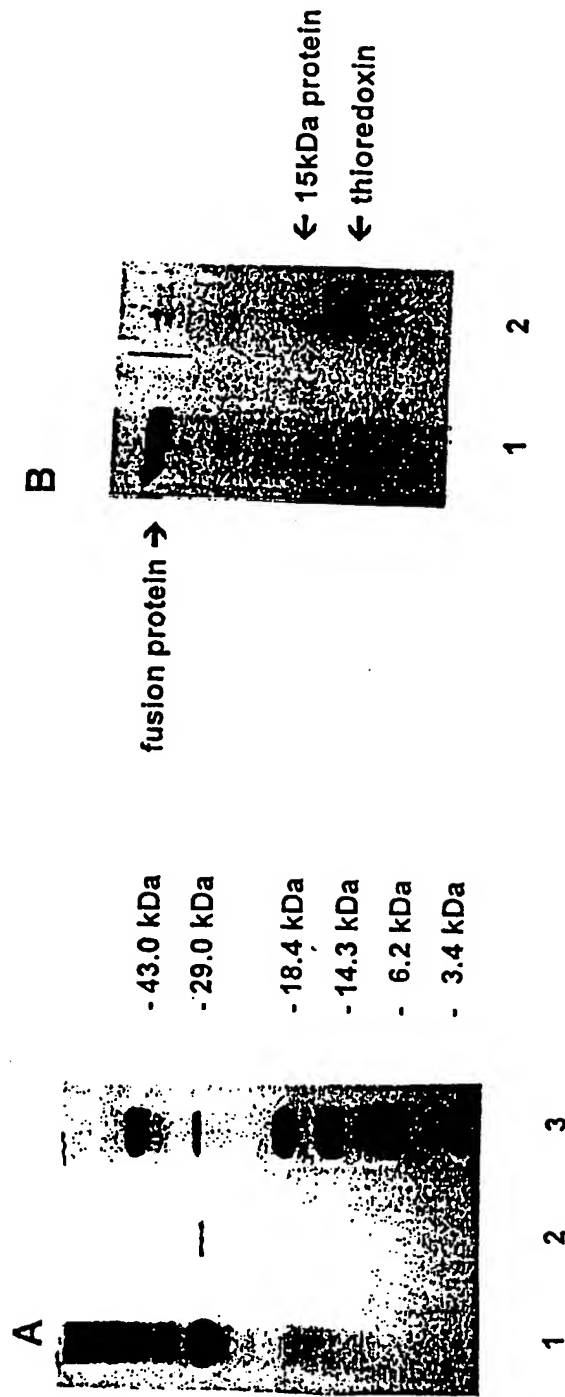


Fig. 8 A: SDS-PAGE of the insoluble pellet fraction (lane1) and the purified ca. 15kDa recombinant antigen (lane2). Lane A3 shows protein molecular weight standards (2.850-43.000 molecular weight range; GIBCO BRL). B: SDS-PAGE of the purified ca. 15kDa thioredoxin fusion protein (lane1) and the two protein bands obtained after enterokinase cleavage (lane2).

10 20 30 40 50
GTCGACGCT ACCGCACCTT CGTCGGCGAG ATGGACGACG AAGAGGCCGA
60 70 80 90 100
CCATCATTAC CGCGCGGGCA TGGCGATGGG CACCACGTTG CAGGTGCCGC
110 120 130 140 150
CGCAGATGTG GCCACCGGAT CGGGCGGCCT TCGACCGCTA CTGGCGGCAA
160 170 180 190 200
TCACTGGACA GGGTGCACAT CGATGACGTC GTTCGCGACT ACCTGTATCC
210 220 230 240 250
GATCGTGGCG CTCCGAATTC GCGGGATCGC ACTGCCGGGT CCGCTGCGGC
260 270 280 290 300
GGCTGTCGGA GGGTATCGCG CTGCTGATCA CCACCGGTTT CCTGCCGCAG
310 320 330 340 350
CGGTTTCGCG ACGAGATGCG GTTGCCGTGG GACGCGACCA AGCAGCGGCG
360 370 380 390 400
CTTTGACCG CTCATGGCCG TGCTGCGCAC GGTGAATCGC CTGATGCCGC
410 420 430 440 450
GGTTGTCCG GGAGTCCCG TTCAACCTGA TGCTCTGGGA CCTGGACCGG
460 470 480 490 500
CGGATGAGGC GCGGGCGCCC GCTGGTGTA TCGACGGCTT CGCGTGGACC
510 520 530 540 550
GATGGCGGTA GACCGCTCGC TAGATTGGCG GGCGAATTG GTGCACAGAG
560 570 580 590 600
GCAAACCGGG CGAAATCCCT ATCCAGGCTC ACCACGGCGC AGTGATGCTC
610 620 630 640 650
CACGGCGATG GCGCCGAGTA CCGCGTCAGG TATCAAGTCG CCCGATGCGT
660 670 680 690 700
CGGCCTCGTC GCAGAGTTTT CGCAGCAGCA CCAGGTGTCT GGGGCCGGGG
710 720 730 740 750
CTTGTCGGAA GGTGATGGGG CTGGGCGTTG ACGGCTTCGA CGAATGCGAA
760 770 780 790 800
TGCATCCGCT CGTGGTGACG GAATCTCGAA GATGCGTCGA TTCGTTGTTA
810 820 830 840 850
GCCGGAGGAA CGACGCCAC ACTAGGTTTC GCACTGTGAA GGGGTCGTCG
860 870 880 890 900
GCCGCAAGCA GTCGATCGAA CCAGGGGCGG ACGGTTCCGT GATTCGGATG
910 920 930 940 950
GTCACCGCGG TGTGCAGCCA GCAGCACGTT GACGTCGATG AGGAACATCG

Fig. 9

1/5

960 970 980 990 1000
CCTATTTGTG CCTGTCCAGG CTCACTTCCG CGAGTTCAGT TCCAGACCCCT
1010 1020 1030 1040 1050
CGTCGAGCAC TTCGGACAAC ACCGTATTTCG AGGTTAGGTC GATACCTGGC
1060 1070 1080 1090 1100
CGCGGACCGG TGCCGGCGTC AAAAACGGGG ACGGTTGGCC GGGCGCCGCC
1110 1120 1130 1140 1150
GGTACGGGCG GCGGCGAGCT CCCGCCGAAG GCGTCTTCG ATCACAGCGC
1160 1170 1180 1190 1200
CCAGCGATTA ACCACGCTCG CGGGCCCGGC GTTTGGCGGT AGCCAGTAGT
1210 1220 1230 1240 1250
TCATCCGAGA TTGACACGGT GGTGCGCATG ATGCTCAGGA TAGCGCATCT
1260 1270 1280 1290 1300
ACGGCATCAT CTGCGGTGAG CAACTGATGC CCTCAACGCC GCGTGTGGTC
1310 1320 1330 1340 1350
GCAGGTCTGC CTGCTATGGC AAGCCGTTGA GTCCGTTCTC GCCGAGCAGC
1360 1370 1380 1390 1400
AGCCCGCCGG TGCCGCCGGC ACCGGGCGTG GCGCCGGCTT TGCCGGCGTT
1410 1420 1430 1440 1450
GGCGCCGTTG CCGCCGTTGC CGATCAGCAC GCGTTGCCG CCGACACCAC
1460 1470 1480 1490 1500
CGCTGCCGCC GGTACCGGCG CCAAACCCGC CGGCAACCCC CGTCACCGCC
1510 1520 1530 1540 1550
GTTGCCGAAC ACCCCGGCGT GGCCACCGTC ACCGCCGGTG CCGCCGGTAC
1560 1570 1580 1590 1600
CGGCGCCTAG AGCGTTGGCA CCGCTGCCGC CGGCGCCGCC GGCGCCGGCG
1610 1620 1630 1640 1650
GAGCCGAAGA GCAAGCCGCC GTTCCCGCCG GCGCCGCCGG CGCCGCCTTG
1660 1670 1680 1690 1700
CTGGATGCTG GTAAGTGCTG CCCGCCCGTG CCCGCCGGCG CCGCCGGCGC
1710 1720 1730 1740 1750
CGCCGAAGCC GAAGAGTAAG GCGCCGTTCC CGCCGGTTCC GCCGGCCCCG
1760 1770 1780 1790 1800
CCGGCAAGGG AGCTGGCGCC ACCGCTGCCG CCGCGCCAC CGGAGGCGCC
1810 1820 1830 1840 1850
GAGGGAGAGT AGGCCGGCGT TGCCGCCGTG CCCGCCGCC CCGGTGGTGA
1860 1870 1880 1890 1900
TCCCGGACCC TCCCGAGCCG GCGGCGCCG CCGTGCCGCC GGCTCCGAAC

Fig. 9
2/5

1910 1920 1930 1940 1950
AGTCCGCCGT TCCCGCCGTT CCCACCGGCC CCGAAGTTCG TGCCGGCCCC
1960 1970 1980 1990 2000
GCCGGTGCCG CCAGTTCGGA ACAGTCCGCC GTTCCCGCCG TTCCCGCCGG
2010 2020 2030 2040 2050
CTGCGTTGAA CCCGCCGGCC CCTCCGGCTC CGCCGTTGGC GAACAGTCCG
2060 2070 2080 2090 2100
CCGTTGCCGC CGGCGCCGCC GACGCCGGCC GGGACACCGC CAGCGGCGCC
2110 2120 2130 2140 2150
GTGGCCGCCG GTGCCGGCCG CGCCGAAGAG CAAACCGGCG TCGCCGCCCG
2160 2170 2180 2190 2200
GCCCCCGGC CCCGCCGATG CCAGCGACGC CTATGGAGTT CCCACCGTTG
2210 2220 2230 2240 2250
CCGCCGGTGC CGCCGGATCC GATCAGCAAG GAGACCCAC CGGCGCCGCC
2260 2270 2280 2290 2300
GGCCCCGCCG ATCCCTCCAG CACCGGTGCC TATCCCGCCG GTCCCGCCAT
2310 2320 2330 2340 2350
TGCCACCGGT ACCGAACAAG ATCCCGCCCG CCCC GCCCGC CCGCCCCGTA
2360 2370 2380 2390 2400
GCCGTGGCGG CGGTGTTGGT CGCACCGTGC CCGCCGTTAC CGCCGTTGCC
2410 2420 2430 2440 2450
GAACAACCAC CCGCCGGCCC CGCCGGCAGC CCCGGTCCCC GGGGTCCCCG
2460 2470 2480 2490 2500
TGCGCGCGTT GCCGAACAGC CACCCGCCCG CCCC GCCCGT AGCCCCGGTT
2510 2520 2530 2540 2550
CCAGGAGTCC CGTTGGCGCC GTTGCCGATC AGCGGGCGGC CGGTGAGCGT
2560 2570 2580 2590 2600
CTGGAAGGGC TCGTTCACCA CATTGAGCAC ATTTTGCTGC AGGGTGTGCA
2610 2620 2630 2640 2650
GTGGCGAGGT GTCGCGGGA GCATTGAATC CGTCTAGACC GAGCAGGAGC
2660 2670 2680 2690 2700
CCGCTGACGA CGACCACTCC GGCCCTTGCCC GCGCCAATCC CACCGCTACC
2710 2720 2730 2740 2750
GCCGTTACCG CCATTGCCGA TCAACACGGC GGTGCCACCG ATCCCGCCGT
2760 2770 2780 2790 2800
TGCCGCCGGT CACCGCGCTG GCGCCACCGT TACCGCCGTT GGCGCCGTTA
2810 2820 2830 2840 2850
CCGATCAGCC CGGGGGTGCC GCCAGCCCCA CCGATCCCGC CGGGGAAGCC

2860 2870 2880 2890 2900
CTGGACAACT CCGCCGTTGG CGCCGGCGCC GCCGGAGCCG AAGACCGTGC
2910 2920 2930 2940 2950
CGGTGTTGCC CCCGGGGCCG TCTTGCCCGC CGTCGGAGAA GCCGAATCCG
2960 2970 2980 2990 3000
CCGGCGCCGC CGGAGCCGCC GGAGCCGAAG AGCAGCCCAG CGTTGCCGCC
3010 3020 3030 3040 3050
GGCGCCGCCG GCGCCGtCTA TGCCGTCGGC CGTGAGAGTA CCGCCGTCCC
3060 3070 3080 3090 3100
CACCGATTCC GCCGGCGCCG CCCGCGGCGC CGAGGGCGAG CATGCCGGCA
3110 3120 3130 3140 3150
TTGCCGCCGG CCCC GCCGTC CCCGCCGGCG ACCAGGCTGT GTCCGCCGCT
3160 3170 3180 3190 3200
GCCGCCTTCC CCGCCTGCGC CGAACAGCCC GCCGGCCCCG CCGGCCCCGC
3210 3220 3230 3240 3250
CGACTCCGCC GAAGCTGCTG TCGGCGAACC CGCCATGCCC GCCGGTGCCG
3260 3270 3280 3290 3300
CCGGCGCCGA ACAGACCGCC AGCGCCACCG GCCCCACCGG CCCC GCCGGA
3310 3320 3330 3340 3350
GCTGCCGGCC CCACCGGATC CGCCGACCCC GCCGGTGGCG AACAGCCCGC
3360 3370 3380 3390 3400
CGGCCCCGCC GCGCGCGCCC GCCCCGCCGA GTGCACTGCC GTTCGTGAAT
3410 3420 3430 3440 3450
CCGCCGGCCC CGCCGACTCC GCGGCGCCCG AAGAGCAGGC CGGCGTTGCC
3460 3470 3480 3490 3500
GGCAGCCCCG CCGGCGCCGC CGGCCCCGCC CGTGAGGGCT ACTACGCCGC
3510 3520 3530 3540 3550
CGCCGGCGCC GCCGGCGCCG CCGGCGCCGA ACAGCATGGC GTTGCCGCCG
3560 3570 3580 3590 3600
GCTCCGCCGG ACCCGCCGAT CCCACTGCTG GCGACCCCGC CAGCGCCGCC
3610 3620 3630 3640 3650
GGCGCCGCCG TTGCCGATGA GCCCGCCGGC GCCGCCGTTG CCGCCGGCCG
3660 3670 3680 3690 3700
CGCCGGATCC TCCGGCGCCG CCGTTGACGA TTAACCAGCC GCCGTCCCCG
3710 3720 3730 3740 3750
CCATTGGCCC CGGTGCCGGG GGCGCCGTTG GCGCCGTTGC CGATCAACGG
3760 3770 3780 3790 3800
GCGCCCGGTA TTCGCCAGGA AGAACTCGTT GATCGGATCC AGCAGCGGCG

3810 3820 3830 3840 3850 Fig. 9
ACACCGCGGC GGCCTCGGCG GCCGCATAGG CGCCGCCACC GGAGGTCAAT 5/5
3860 3870 3880 3890 3900
GCCTGCACGA ACTGGGCATG AAACGCCTGC GCTTGGGCGC TGAGCGCCTG
3910 3920 3930 3940 3950
ATAGGCCTGG CCGTGGGCGC CGAACAGCGC GGCGATGGCT GTCGAC

17/23

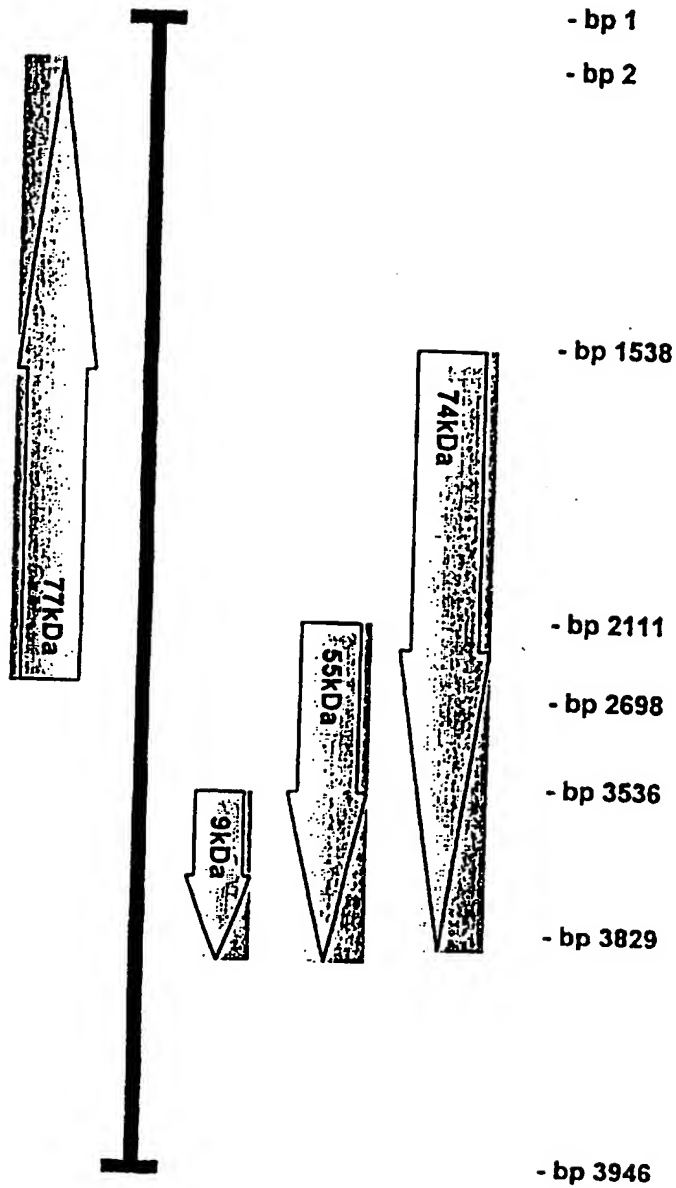


Figure 10. Schematic drawing of the clone Mtub-Clara-Klon. The open reading frames for about 9kDa (bp 3536 - bp 3829), 55kDa (bp 2111 - bp 3829), 74 kDa (bp 1538 - bp 3829) and 77 kDa (bp 2698 - bp 2 on the complimentary strand) proteins are shown by arrows and the corresponding coding regions are numbered.

18/23

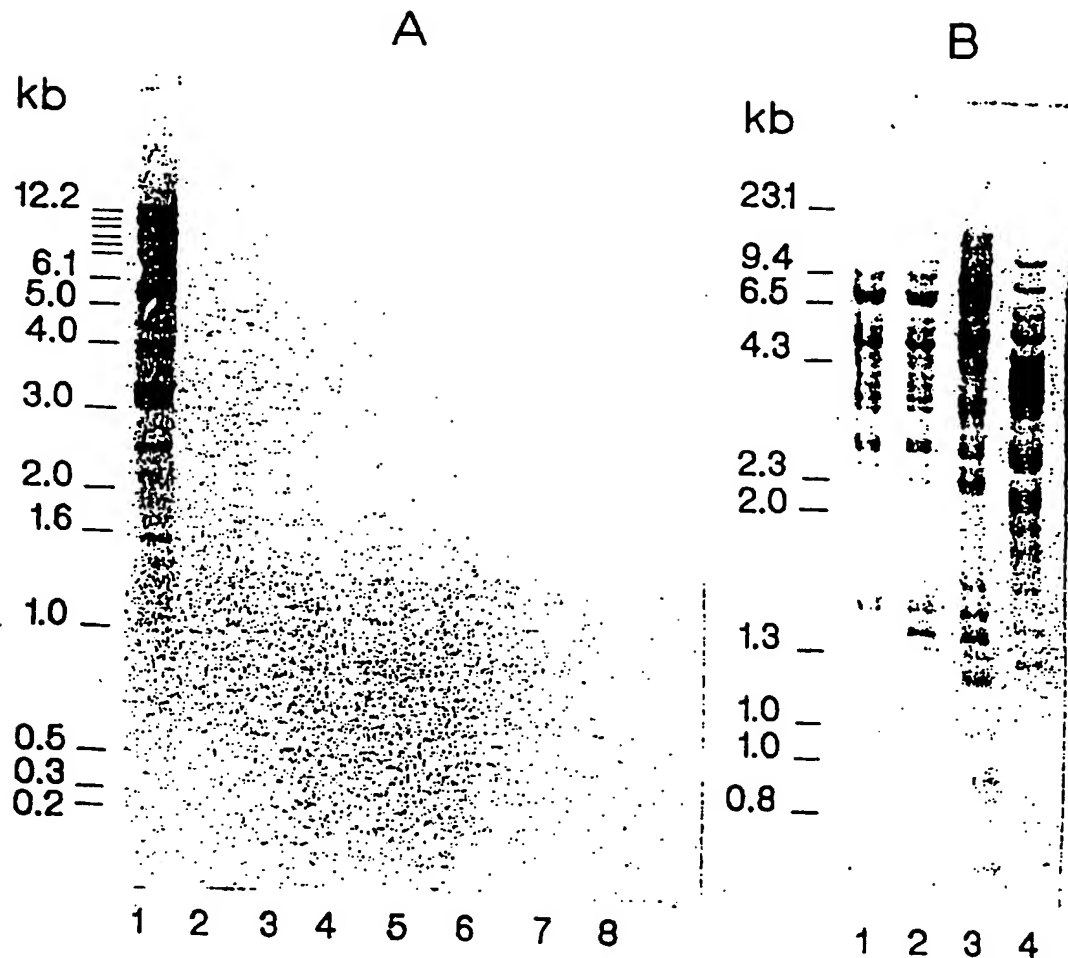


Fig. 11 A. Southern hybridization with genomic DNA from different mycobacteria digested with PvuII (1. *M. tuberculosis* H37Rv, 2. *M. avium*, 3. *M. kansasii*, 4. *M. necroti*, 5. *M. fortuitum*, 6. *M. phlei*, 7. *M. smegmatis*, 8. *M. vaccae*.)

Fig. 11B

Finger-print obtained using the DNA (BamHI digest) of 1. *M. tuberculosis* H37 RV, 2. *M. tuberculosis* H37 Ra, 3. *M. bovis* BCG, and 4. *M. tuberculosis* H37Rv digested with Sal I.

19/23

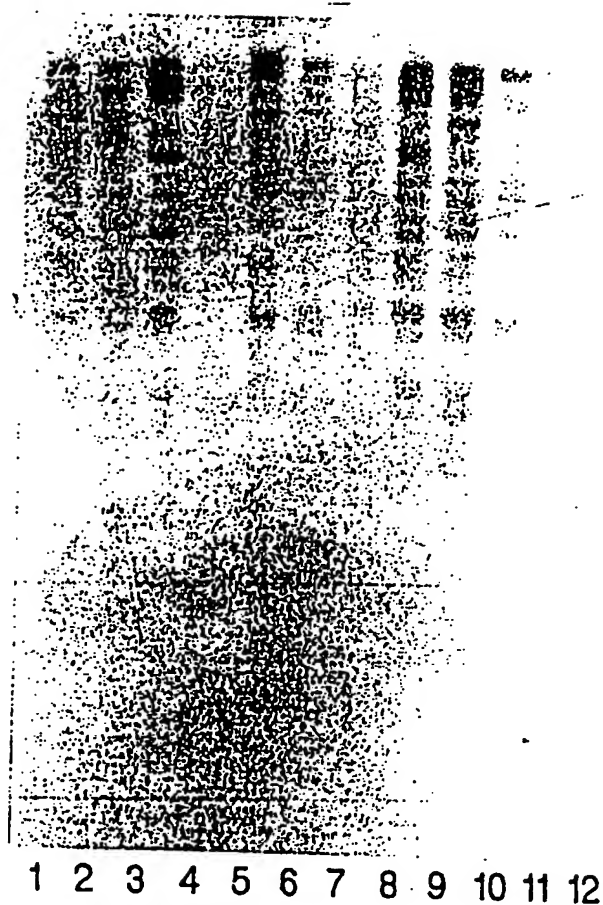


Fig 12. Finger-print with DNA from different *M. tuberculosis* clinical isolates (numbered 1- 12) digested with PvuII restriction enzyme. The 4 Kb Sal I fragment (Mtub-Klar-Klon) was used as probe.

Fig. 13

20/23

Amino acid sequence of the protein of about 74kDa.

Molecular-weight 74999 ; Length 764

THE AMINO ACID SEQUENCE IS GIVEN BELOW:

10	20	30	40	50	60
VPPVPAPRAL	APLPPAPPAP	AEPKSKPFFP	PAPPAPPCWM	LVSAAPPCPP	APPAPPKPKS
70	80	90	100	110	120
KAPFFPVPPA	PPARELAPPL	PPAPPEAPRE	SRPALPPCPP	PPVVIPDPPE	PAAPPVPPAP
130	140	150	160	170	180
NSPFFPPFFP	APKFVPAPPV	PPVPNSPPFP	PFPPAALNPP	APPAPPLANS	PPLPPAPPTP
190	200	210	220	230	240
AGTPPAAPWP	PVPAAPKSKP	ASPPRPPAPP	MPATPMEFPP	LPPVPPDPIS	KETPPAPPAP
250	260	270	280	290	300
PIPPAPVPIP	PVPELPPVPN	KIPPAPPAPP	VAVAAVLVAP	CPPLPPLPNN	HPPAPPAAPV
310	320	330	340	350	360
PGVPLAPLPN	SHPPAPPSAP	VPGVPLAPLP	ISGRPVSVWK	GSFTTLSTFC	CRVCSGEVLA
370	380	390	400	410	420
GALNPSRPSR	SPLTTTTPAL	PAPIPPLPPL	PPLPINTAVP	PIPPLPPVTA	LAPPLPPLAP
430	440	450	460	470	480
LPISPGVPPA	PPIPPGKPWT	TPPLAPAPPE	PKTVPVLPFG	PSCPPSEKPN	PPAPPEPPEP
490	500	510	520	530	540
KSSPALPPAP	PAPSMPSAVR	VPPSPPIPPA	PPAAPRASMP	ALPPAPPSPP	ATRLCPPLPP
550	560	570	580	590	600
SPPAPNSPPA	PPAPPTPKL	LSANPPCPPV	PPAPNRPPAP	PAPPAPPELP	APPDPPTPPV
610	620	630	640	650	660
ANSPPAPPAP	PAPPSALPFV	NPPAPPTPAA	PKSRPALPAA	PPAPPAPPVR	ATTPPPAPPA
670	680	690	700	710	720
PPAPNSMALP	PAPPDPPIPL	LATPPAPPAP	PLPMSPPAPP	LPPAAPDPPA	PPLTINQPPS
730	740	750	760	770	780
PPLAPVPGAP	LAPLPINGRP	VFARKNSLIG	SSSGDTAAAS	AAA*.....

Fig. 14

21/23

A glycine rich protein of about 77kDa.

Molecular-weight 77056 ; Length 899

THE AMINO ACID SEQUENCE IS GIVEN BELOW:

10	20	30	40	50	60
STAIAALFGA	HGQAYQALSA	QAQAFHAQFV	QALTSGGGAY	AAAEAAVSP	LLDPINEFFL
70	80	90	100	110	120
ANTGRPLIGN	GANGAPGTGA	NGGDGGWLIV	NGGAGGSGAA	GGNGGAGGLI	GNGGAGGAGG
130	140	150	160	170	180
VASSGIGGSG	GAGGNAMLFG	AGGAGGAGGG	VVALTGGAGG	AGGAAGNAGL	LFGAAGVGGA
190	200	210	220	230	240
GGFTNGSALG	GAGGAGGAGG	LFATGGVGGG	GGAGSSGGAG	GAGGAGGLFG	AGGTGGHGGF
250	260	270	280	290	300
ADSSFGGVGG	AGGAGGLFGA	GGEAGSGGHS	LVAGGDGGAG	GNAGMLALGA	AGGAGGIGGD
310	320	330	340	350	360
GGTLTADGID	GAGGAGGNAG	LLFGSGGSGG	AGGFGFSDGG	QDGPGGNTGT	VFGSGGAGAN
370	380	390	400	410	420
GGVVQGFPGG	IGGAGGTPGL	IGNGANGGNG	GASAVTGGNG	GIGGTAVLIG	NGGNGGSGGI
430	440	450	460	470	480
GAGKAGVVVV	SGLLLGLDGF	NAPASTSPLH	TLQQNVNLNV	NEPFQTLTGR	PLIGNGANGT
490	500	510	520	530	540
PGTGADGGAG	GWLFGNGANG	TPGTGAAGGA	GGWLFNGNGN	GGHGATNTAA	TATGGAGGAG
550	560	570	580	590	600
GILFGTGGNG	GTGGIGTGAG	GIGGAGGAGG	VSLLIGSGGT	GGNGGNSIGV	AGIGGAGGRG
610	620	630	640	650	660
GDAGLLFGAA	GTGGHGAAGG	VPAGVGGAGG	NGGLFANGGA	GGAGGFNAAG	GNGGNGGLFG
670	680	690	700	710	720
TGGTGGAGTN	FGAGGNGGNG	GLFGAGGTGG	AAGSGGSGIT	TGGGGHGGNA	GLLSLGASGG
730	740	750	760	770	780
AGGSGGASSL	AGGAGGTGCG	CALLFGFGGA	CGACGIGGAA	LTSIQGGAG	GAGGNGGLLF
790	800	810	820	830	840
GSAGAGGAGG	SGANALGAGT	GGTGGDGGHA	GVFGNGGDGG	CRRVRRRYRR	QRWCRRQRRR
850	860	870	880	890	900
DRQRRQRRQR	RQSRGHARCR	RHRRAAARRE	RTQRLAIAGR	PATTRGVEGI	SCSPQMMP*

Fig. 15

22/23

Amino acid sequence of the about 9 kDa proline
rich protein

Molecular-weight 9356 ; Length 98

AMINO ACID SEQUENCE IS GIVEN BELOW:

10	20	30	40	50	60
MALPPAPPDP	PIPLLATPPA	PPAPPLPMSP	PAPPLPPAAP	DPPAPPLTIN	QPPSPPLAPV
70	80	90	100	110	120
PGAPLAPLPI	NGRPVFARKN	SLIGSSSGDT	AAASAAA*..

23/23

Fig. 16

Proline rich protein of about 55kDa.
 Molecular-weight 55982 ; Length 573
 AMINO ACID SEQUENCE IS GIVEN BELOW.

10	20	30	40	50	60
VPAAPKSKPA	SPPRPPAPPM	PATPMEFPPL	PPVPPDPISK	ETPPAPPAPP	IPPAPVPIPP
70	80	90	100	110	120
VPPLPPVPNK	IPPAPPAPPV	AVAAVLVAPC	PPLPPLPNNH	PPAPPAAPVP	GVPLAPLPNS
130	140	150	160	170	180
HPPAPPSAPV	PGVPLAPLPI	SGRPVSVWKG	SFTTLSTFCC	RVCSGEVLAG	ALNPSRPSRS
190	200	210	220	230	240
PLTTTTPALP	APIPPLPPLP	PLPINTAVPP	IPPLPPVTAL	APPLPPLAPL	PISPGVPPAP
250	260	270	280	290	300
PIPPGKPWTT	PPLAPAPPEP	KTVPVLPVGP	SCPPSEKPNP	PAPPEPPEPK	SSPALPPAPP
310	320	330	340	350	360
APSMPSAVRV	PPSPPIPPAP	PAAPRASMPA	LPPAPPSPPA	TRLCPPPLPS	PPAPNSPPAP
370	380	390	400	410	420
PAPPTPPKLL	SANPPCVPV	PAPNRPPAPP	APPAPPELPA	PPDPPTPPVA	NSPPAPPAPP
430	440	450	460	470	480
APPSALPFVN	PPAPPTPAAP	KSRPALPAAP	PAPPAPPVRA	TTPPPAPPAP	PAPNSMALPP
490	500	510	520	530	540
APPDPPPIPL	ATPPAPPAPP	LPMSPPAPPL	PPAAPDPPAP	PLTINQPPSP	PLAPVPGAPL
550	560	570	580	590	600
APLPINGRPV	FARKNSLIGS	SSGDAAASA	AA*.....